In focus in HCB

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In this last issue of the journal for 2021, we will highlight three original articles describing (1) an ultrastructural analysis of the effects of Zika virus infection on cultured glioblastoma cells; (2) the role of cytokines on rat testicular macrophages, Leydig cell steroidogenesis, and seminiferous tubule integrity; (3) a comparison of formalin-fixed paraffin-embedded (FFPE) processed tissues and an alcohol-based fixative (PAXgene) for routine diagnostic histology, immunohistochemistry, and molecular analyses; and one short communication analyzing transepithelial electrical resistance (TEER) measurements during Caco-2 cell differentiation in culture. We hope you enjoy these brief highlights along with the entire issue, and wish all of our readers “Happy Holidays” and a safe and healthy New Year.

Potential therapeutic treatment for glioblastoma goes viral

Zika virus, a member of the *Flaviviridae* family transmitted to humans via a mosquito bite, has recently been proposed as a potential therapeutic treatment for malignant glioblastoma due to its targeting and oncolytic action towards glioblastoma stem cells (Francipane et al. 2021). However, prior to its potential use in clinical trials, a more detailed analysis of the effects of the virus on glioblastoma cells must be undertaken. Wieland et al. (2021) have now provided such an analysis of the effects of Zika virus infection on the ultrastructural appearance of two glioblastoma cell lines using state-of-the-art high-pressure freezing/freeze-substitution

followed by conventional transmission electron microscopy and scanning transmission electron microscopy (STEM) tomography. The gold standard technical processing protocol in this field calls for rapid freezing of unfixed cells (Walther et al. 2018); however, due to safety protocols, Zika-infected cells must first be treated with glutaraldehyde to render the virus inactive. The authors therefore examined uninfected glioblastoma cells with and without aldehyde fixation prior to high-pressure rapid freezing, and found that the aldehyde treatment did not have any negative effects on the Golgi apparatus ultrastructure, an organelle likely involved in virus replication. They then proceeded to investigate the ultrastructural appearance of the cytoplasm of two Zika virus-infected glioblastoma cell lines (Fig. 1).

Fig. 1 In the Zika virus-infected glioblastoma cell line #15747GB, viral capsids (red) were observed in endoplasmic reticulum (ER) cisternae at a large distance from the replication organelles (yellow). From Wieland et al. (2021)

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Their beautiful and high-quality conventional electron microscopy images and STEM tomography provided the following results: (1) very interestingly, Golgi apparatus structures were not visible in the infected cell lines; (2) two different types of cytoplasmic viral factories were observed—type 1, containing densely packed replication organelles, which are luminal vesicles inside the endoplasmic reticulum (ER), and type 2, which contained fewer replication organelles, but were swollen ER segments with attached ribosomes; (3) cytoplasmic ultrastructural features differed between the two glioblastoma cell lines; (4) “string-like” filaments observed near replication organelles may represent viral ribonucleoproteins in transport to viral particles; and finally, (5) cellular ultrastructural alterations caused by Zika virus (where the viral particles are assembled in the ER) are much different from those caused by a phlebovirus where the viral particles are assembled in the Golgi apparatus. This highly detailed analysis provides further insight into the ultrastructural effects of Zika virus infection and subsequent replication on glioblastoma cells, necessary information in the assessment of its potential oncolytic therapeutic use.

Testicular function and cytokines

Under physiological conditions, cytokines seem to play a role in the function of the testes (Svechnikov et al. 2003; Warren et al. 1990). There is evidence that certain cytokines, including interleukin-1 and interleukin-6, tumor necrosis factor- α , and macrophage migration inhibitory factor, are important for normal functioning of Leydig cells, Sertoli cells, testicular macrophages, and germ cells (Gerendai et al. 2005; Syed et al. 1995; Verhoeven et al. 1988; Warren et al. 1990). In their present work, de Oliveira et al. (2021) have treated rats with diacerein, an anti-inflammatory drug that inhibits interleukin-1 (Abdel-Gaber et al. 2018; Pasin et al. 2010; Tamura and Ohmori 2001), and tumor necrosis factor- α (Pasin et al. 2010) to obtain further information of the impact these cytokines may have on testicular function. The parameters they analyzed following 30 days of treatment with diacerein included testicular macrophages, Leydig cell steroidogenesis, and seminiferous tubule integrity. Specifically, they measured the serum testosterone levels and the seminiferous tubule area, and evaluated the epithelial area, frequency of damaged seminiferous tubules, and the number of Sertoli cells. By immunohistochemistry and Western blotting, pro-interleukin-1 β , tumor necrosis factor- α , steroidogenic acute regulatory protein, 17 β -hydroxysteroid dehydrogenase, androgen receptor, and scavenger receptor for hemoglobin-haptoglobin complexes (CD163), a testicular macrophage marker, were analyzed (Fig. 2).

Fig. 2 Schematic presentation of the changes in Leydig cells and ED2 testicular macrophages induced by diacerein. From de Oliveira et al. (2021)

Initially, they confirmed that diacerein treatment reduced interleukin-1 and tumor necrosis factor- α immunoreactivity in the interstitial tissue and seminiferous tubules. This resulted in various pathological changes. The total tubular and epithelial areas were significantly reduced. This was obviously due to an increased apoptosis rate of germ cells and Sertoli cells. Furthermore, interstitial macrophages were significantly decreased. In regard to Leydig cells, steroidogenesis was found to be impaired, as indicated by reduced immunolabeling for 17 β -hydroxysteroid dehydrogenase and steroidogenic acute regulatory protein, and reduced levels for 17 β -hydroxysteroid dehydrogenase in testicular extracts as shown by Western blots. This was accompanied by reduced serum testosterone levels. It is known that CD163-positive testicular macrophages are involved in the control of steroidogenesis and that they exert paracrine effects on Leydig cells. Their reduced number in diacerein-treated rats furthermore points to the importance of Leydig cell–macrophage interaction. Taken together, the results for the seminiferous tubules obtained after diacerein-induced cytokine deficiency provide further evidence for a role of Sertoli cells in androgenic control.

An alternative to formalin fixation for better preservation of tissue nucleic acids

Formalin, a saturated 37% aqueous solution of formaldehyde, has been used as a tissue fixative for anatomy and histology since the mid-1890s (Musial et al. 2016). Indeed, its molecular cross-linking properties for a variety of biological molecules have made it the fixative of choice for clinical diagnostic laboratories. Although it maintains tissue histological preservation, as routinely observed on sections of FFPE samples stained with hematoxylin and eosin, as well as many tissue antigens revealed through immunohistochemistry, its detrimental effect on nucleic acids renders modern molecular diagnostic assays problematic with such

samples. Given this issue, as well as its well-known toxic properties and environmentally hazardous waste disposal concerns, searches for alternative tissue fixatives or minimal-use formalin methods for clinical laboratories have been undertaken for many years (Gatta et al. 2012; Mastrocchi et al. 2019). To this end, Meecham et al. (2021) now present results from a study involving human tissue samples obtained at three laboratory sites in the United Kingdom, comparing routinely processed FFPE samples to a novel alcohol-based fixative (PAXgene Tissue) by analyzing tissue morphology, immunohistochemistry, fluorescence in situ hybridization (FISH), and DNA and RNA preservation (Fig. 3).

Fig. 3 The DNA yield from PAXgene-fixed samples and FFPE samples was not significantly different. However, the DNA fragment length from PAXgene-fixed samples was significantly greater. From Meecham et al. (2021)

The results of this comparative study showed varied opinion amongst the three laboratory sites and tissue origins for both of the tissue fixation protocols. However, in comparing the FFPE results with the PAXgene Tissue results, the following details were noted: (1) histological analysis revealed no significant differences; (2) immunohistochemistry showed a general diminished staining intensity in the PAXgene-fixed samples compared to the FFPE samples; (3) FISH results were comparable between the two fixation protocols; (4) DNA fragment length and integrity numbers were significantly greater in the PAXgene-prepared samples compared with those from FFPE; and (5) although FFPE samples provided higher RNA yields than the PAXgene-preserved samples, RNA purity and the RNA integrity number (RIN) were improved with PAXgene- versus FFPE-fixed

samples. Overall, the results indicated that PAXgene tissue fixation may provide an alternative tissue preservation method in diagnostic pathology when molecular information is needed for accurate diagnosis. Further analyses across a wide range of testing sites, tissues, and methods will be necessary to establish the potential replacement of routine FFPE processing with an alcohol-based fixative such as PAXgene.

Transepithelial electrical resistance in monolayer cultures is determined by junctional length and differentiation

The barrier integrity of monolayers of various types of cultured epithelial and endothelial cells is commonly measured by the transepithelial electrical resistance (TEER). Since TEER measurements are nondestructive, they enable time-course studies to monitor dynamic changes in the cellular barrier function (Rezaee and Georas 2014; Schlegel et al. 2012; Srinivasan et al. 2015). In their previous in vitro work on the mechanisms involved in the regulation of intestinal barrier function, Meir et al. (2015) and Spindler et al. (2015) observed a great variation in absolute TEER values, especially during the early phase after confluence. Other investigators have observed wide variation in TEER as well, which remains to be fully explained (Blume et al. 2010; Briske-Anderson et al. 1997; Robilliard et al. 2018). In their present work, Felix et al. (2021) measured the TEER in monolayers of Caco-2 clone 1 and clone 2 cells during their differentiation, paying special attention to changes in the junctional complex and the overall cell morphology within monolayers. For the analyses, they used immunocytochemistry to visualize E-cadherin, desmoglein-2, and claudin-1 and 5, and quantified both the cell number and the circumferential junctional length (Fig. 4). In addition, they measured the flux of 4 kDa fluorescein isothiocyanate (FITC)-dextran across the cell monolayers.

Fig. 4 A plot showing the changes in TEER and corresponding junctional length in clone 2 Caco-2 cells from confluence (green) over differentiating barrier (red) to full barrier function (blue). From Felix et al. (2021)

For both clonal cell lines they observed a similar behavior, an increased TEER until confluence, followed by a temporal decrease during the differentiating barrier stage, and another increase in the TEER at fully differentiated barrier. This was paralleled by a continuous reduction in the permeability of 4 kDa FITC–dextran. Regarding the observed changes in the TEER, the authors conclude that the TEER is dominated by the growing number of cells and thus the junctional length during early stages of maturation, whereas at later stages it is the continuous strengthening of the barrier that results in paracellular resistance and a rise in TEER values. The authors therefore recommend a thorough and parallel assessment of the morphology and maturation state of junctions when performing barrier measurements by TEER.

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